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Autocrine Regulation of Gonadotropin-releasing Hormone (GnRH) Operates at Multiple Control Levels of GnRH Gene Expression in GT1-1 Neuronal Cells

Jin Han, Sehyung Cho, Woong Sun, and Kyungjin Kim*

Department of Molecular Biology and Research Center for Cell Differentiation, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea

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We previously found that a potent gonadotropin-releasing hormone (GnRH) agonist, buserelin, decreases GnRH promoter activity together with GnRH mRNA level, providing evidence for an autoregulatory mechanism operating at the level of GnRH gene transcription in immortalized GT1-1 neuronal cells. To examine whether agonist-induced decrease in GnRH mRNA level requires the continuous presence of buserelin, we performed a pulse-chase experiment of buserelin treatment. Short-term exposure (15 min) of GT1-1 neuronal cells to buserelin (10 μM) was able to decrease GnRH mRNA levels when determined 24 h later. When GT1-1 cells were treated with buserelin (10 μM) for 30 min and then incubated for 1, 3, 6, 12, 24, and 48 h after buserelin removal, a significant decrease in GnRH mRNA levels was observed after the 12 h incubation period. These data indicate that inhibitory signaling upon buserelin treatment may occur rapidly, but requires a long time (at least 12 h) to significantly decrease the GnRH mRNA level. To examine the possible involvement of *de novo* synthesis and/or mRNA stability in buserelin-induced decrease in GnRH gene expression, actinomycin D (5 μg/ml), a potent RNA synthesis blocker, was co-treated with buserelin. Actinomycin D alone failed to alter basal GnRH mRNA level, but blocked the buserelin-induced decrease in GnRH mRNA level at 12 h of post-treatment. These data suggest that buserelin may exert its inhibitory action by altering the stability of GnRH mRNA. Moreover, a polysomal RNA separation by sucrose gradient centrifugation demonstrated that buserelin decreased the translational efficiency of the transcribed GnRH mRNA. Taken together, these results clearly indicate that GnRH agonist buserelin acts as an inhibitory signal at multiple levels such as transcription, mRNA stability, and translation.

Gonadotropin-releasing hormone (GnRH) is a pivotal neuropeptide controlling pituitary function. Its secretion and gene expression are influenced by a variety of neural and humoral substances (Knobil, 1980; Merchenthaler et al., 1984; Stojilkovic et al., 1994; Gore and Roberts, 1997; Kim et al., 1997). GnRH secreted from its nerve terminals may exert an inhibitory action on its own secretion. A possible autoregulatory mechanism for this neuropeptide has been supported by several findings. For example, GnRH concentrations in hypophyseal portal plasma were reduced by GnRH receptor agonist in ovariectomized rats (Valenca et al., 1987). GnRH or its agonist was also found to inhibit its own secretion from medial basal hypothalamic fragments incubated *in vitro* (DePaolo et al., 1987). It has been shown that GnRH receptors are expressed in the hypothalamus and in hypothalamic GT1 neuronal cells (Krsmanovic et al., 1993; Jennes and Conn, 1994). It appears then that autocrine action of this peptide may be mediated by its own receptors. Recently, we have shown that treatment of GT1-1 cells with buserelin, a potent GnRH agonist, decreases GnRH mRNA levels and GnRH promoter activity, indicating that autocrine regulation of GnRH operate at the level of GnRH gene transcription (Cho et al., 1997). The present study further explored the possible involvement of post-transcriptional regulation of GnRH by buserelin in GT1-1 neuronal cells.

* To whom correspondence should be addressed.
Tel: 82-2-880-6694, Fax: 82-2-872-1993
E-mail: kyungjin@plaza.snu.ac.kr

Materials and Methods

Materials

Buserelin ([Ser(*t*-Bu)^6, Des-Gly^{10}]-GnRH ethylamide) was obtained from Hoechst (Frankfurt am Main,

Germany). Materials for cell culture were from Gibco (NY, USA) and other chemicals, if not mentioned, were all from Sigma Co. (St. Louis, Mo., USA).

Cell culture

GT1-1 cells were grown on 100-mm culture dishes (Falcon) and maintained in Dulbecco's Modified Eagle's Medium supplemented with 5% fetal bovine serum, 5% horse serum, 4.5 mg/ml glucose (Gibco), 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (Sigma) (Liposits et al., 1991; Wetsel et al., 1992). For Northern blot analysis and polysomal RNA isolation, GT1-1 cells (2×10^6 cells) were re-plated on 6-well culture plates (Falcon) and incubated at 37℃ for 3-4 d in a humidifying incubator in an atmosphere of 5% CO_2 and 95% air until they reached 70% confluence. Prior to stimulation with various substances, cells received fresh media and were incubated for 12-16 h before treatment.

Northern blot hybridization analysis

Detailed procedure for northern blot hybridization was described elsewhere (Seong et al., 1993). Briefly, total RNAs from cells or tissues were extracted by acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Ten μg of total RNAs were denatured, resolved in an 1.2% formaldehyde gel, and transferred to a nylon membrane (Schleicher & Schuell). Membranes were prehybridized at 42℃ for 2 h and then hybridized for 16-24 h with ^{32}P-labeled specific probes as described below. After autoradiography, membranes were rehybridized with 18S cDNA probes. Relative mRNA levels were normalized with 18S RNA signals by quantitative densitometric scanning of the autoradiograms (Hoeffer Scientific Instruments). The probe used to detect the mouse GnRH mRNA in GT1-1 cells was a 396 bp complementary DNA fragment which is identical to 8-403 bp of the mouse GnRH cDNA coding sequence (Mason et al., 1986). This fragment was amplified by conventional reverse transcription-polymerase chain reaction and cloned in to a pGEM-4Z vector (Promega) at the *Sma*I restriction site (pGEM-mGnRH). Sequence identity was confirmed by dideoxy sequencing.

Polysomal RNA isolation

To examine the translational efficiency of the transcribed GnRH mRNA, a polysomal RNA separation by sucrose gradient centrifugation was used (Gore et al., 1995). Briefly, cells were subjected to homogenization with lysis buffer (15 mM, pH 7.5, 60 mM KCl, 15 mM NaCl, 0.3 M sucrose, 0.5% NP-40). The homogenates were centrifuged at 16,000xg for 30 min. This postmitochondrial supernatant was layered over a continuous 10-40% sucrose gradient in a 14x89-mm polyallomer ultracentrifuge tube (Beckman). For the EDTA treatment, EDTA was treated to the fraction at a final concentration of 100 mM and incubated at 30℃ for 10

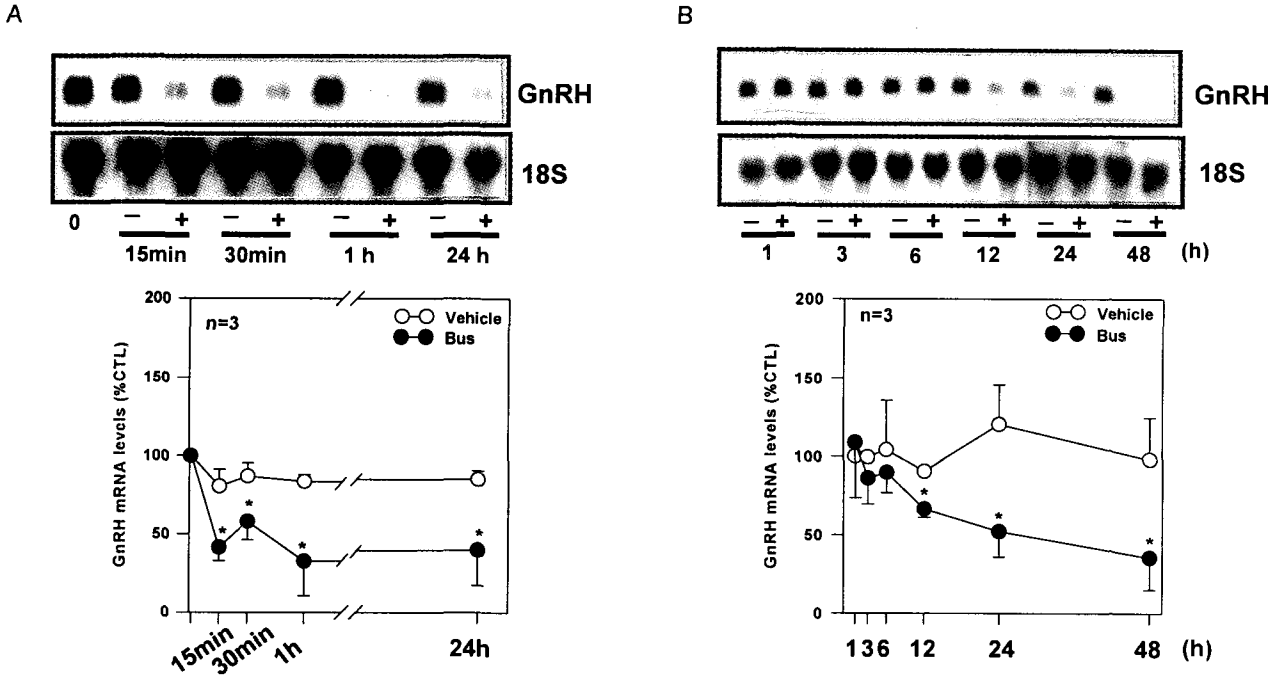

Fig. 1. Effect of short-term exposure of GT1-1 cells to buserelin (10 μM) on GnRH mRNA level. A, GT1-1 cells were treated with 10 mM buserelin or vehicle for 15 min, 30 min, 1 h, and 24 h and harvested 24 h post-treatment. B, GT1-1 cells were treated with 10 mM buserelin or vehicle for 30 min and harvested at 1, 3, 6, 12, 24, and 48 h as indicated. *P<0.05 vs control group. Experiments were repeated three times.

min before layering over the sucrose gradient. The gradients were centrifuged at 100,000xg for 4 h at 4℃ in a SW41 rotor (Beckman). After the centrifugation, RNA was fractionated and precipitated with ethanol. These fractions were centrifuged and the RNA was extracted by the acid guanidinium thiocyanatephenol-chloroform method (Chomczynski and Sacchi, 1987). Ethanol aliquots of purified RNA were electrophoresed on a 1% denaturing agarose gel and stained with ethidium bromide. Gels were photographed and transferred onto the nylon membrane.

Statistical analysis

Statistical comparison between control and experimental groups was performed by one-way analysis of variance (ANOVA) followed by Fisher's least significant difference test for a *post-hoc* comparison. Probability below 0.05 was regarded as statistically significant.

Results

Brief exposure of GT1-1 cells to buserelin is sufficient for the long-term decrease of GnRH mRNA level in GT1-1 cells

To elucidate the time-course effect of buserelin on GnRH mRNA level, a pulse-chase experiment was performed. Previously, we found that a short-term exposure of GT1-1 cells to buserelin for 1-5 min was unable to modify GnRH mRNA levels (Cho et al., 1997). GT1-1 cells were treated with vehicle (0.01% benzyl alcohol) or 10 μM of buserelin for 15 min, 30 min, 1 h, and 24 h before harvesting cells 24 h after buserelin treatment. A significant decrease in GnRH mRNA level was observed in groups treated with buserelin for more than 15 min (Fig. 1A). The short-term exposure of GT1-1 cells to buserelin did not weaken the inhibitory effect of buserelin on GnRH mRNA level. Thus, chronic incubation with buserelin is not indispensable to exert its inhibitory effect, but rather an initial event in response to buserelin might be important to exert the inhibitory effect of buserelin. When cells were treated with 10 μM of buserelin for 30 min and then incubated without buserelin for 1 to 48 h, GnRH mRNA level was significantly ($p < 0.05$) decreased after 12 h of incubation, and further suppressed until 48 h of incubation (Fig. 1B). The relatively prolonged lag period suggests that the molecular action mechanism may be due in part to an indirect activation of other gene(s).

Buserelin decreases stability of GnRH mRNA in GT1-1 cells

To gain insight into the action mechanism underlying buserelin-induced inhibition of GnRH gene expression, the effect of actinomycin D, a *de novo* transcription blocker (Sobell, 1985), on GnRH mRNA level was

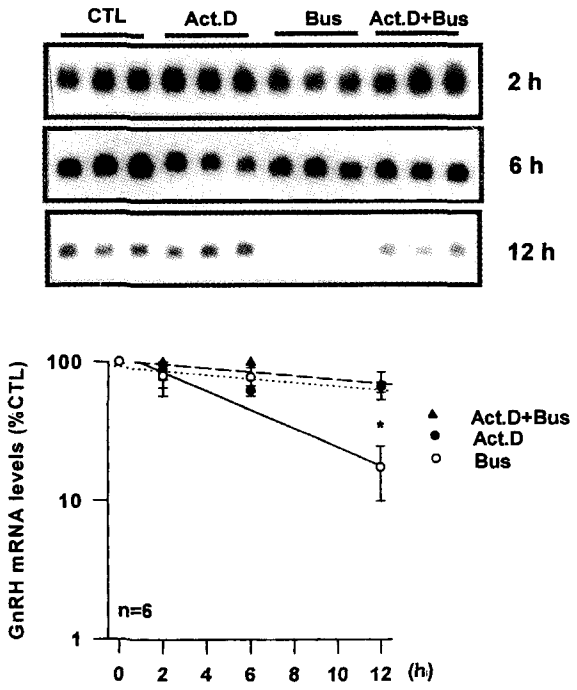

Fig. 2. Effect of co-treatment of GT1-1 cells with transcription blocker (actinomycin D) on buserelin-induced decrease in GnRH mRNA level. GT1-1 cells were treated with vehicle (CTL), 10 μM buserelin (Bus), 5 μg/ml actinomycin D (Act.D) or actinomycin D+buserelin (Act.D+Bus) for 2, 6, 12 h and analyzed for GnRH mRNA level. *P<0.05 vs Act.D+Bus treated group. Experiments were repeated six times.

assessed. GT1-1 cells were treated with vehicle, 10 μ M buserelin, 5 μg/ml actinomycin D, or actinomycin D + buserelin for 2, 6, and 12 h (Fig. 2). When compared with the actinomycin D-treated group, the buserelin-treated group resulted in a drastic decrease in GnRH mRNA level. GnRH mRNA has a long half-life (22-30 h) under basal conditions as reported by others (Bruder and Wierman, 1994; Gore et al., 1997). Yet, GnRH mRNA level decreased more than 50% within 12 h following treatment with buserelin. Thus, it appears that an additional mechanism such as a decrease in the stability of GnRH mRNA, is most likely to occur in response to buserelin treatment.

Buserelin decreases translational efficiency of GnRH mRNA in GT1-1 cells

To test whether buserelin may also affect the translational efficiency of GnRH mRNA species, a distribution of GnRH mRNA in polysomal RNA separation was examined in control and buserelin-treated groups. Fig. 3A shows the representative ethidium bromide staining photograph where RNAs fractionated by sucrose gradient centrifugation were resolved on agarose gel electrophoresis. In the control and buserelin-treated groups (upper and middle panels), the lightest fraction (lane 1) of each gradient contained transfer RNA (tRNA) and other small RNAs, and 18S and 28S ribosomal RNAs

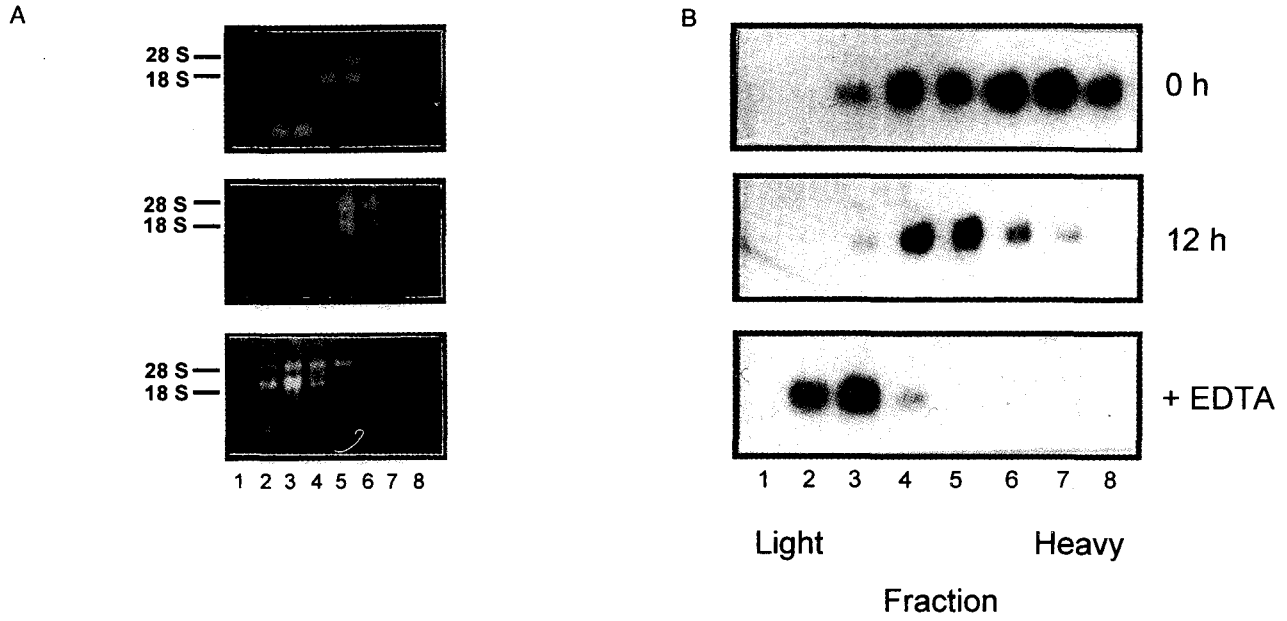

Fig. 3. Negative regulation of translational efficiency of GnRH mRNA by 12 h of buserelin treatment. A, Ethidium bromide staining of RNA extracted from fractionated polyribosomes through a representative agarose gel. Lane 1 indicated the lightest fraction and lane 8 the heaviest. B, A representative autoradiogram of Northern blot analysis showing the distribution of GnRH mRNA.

in lane 2. No difference in the distribution of RNAs between the two groups was observed. On the other hand, EDTA (100 mM) treatment shifted the 18S and 28S ribosomal RNAs into lighter fractions, showing that 40S and 60S ribosomal subunits had been dissociated (lower panel) (Gore et al., 1995).

Northern blot analysis of fractionated total RNA revealed that buserelin treatment caused a shift in the distribution of ribosome-associated GnRH mRNA through the sucrose gradient (Fig. 3B). Most of GnRH mRNA was found in lanes 6-8 in the control group, whereas the peak GnRH mRNA was observed in lanes 4-5 in the 12 h buserelin-treated group (Fig. 3B). To note is that the total amount of GnRH mRNA decreased in buserelin-treated groups, which correlates well with our previous study (Cho et al., 1997). EDTA treatment caused a shift of peaks to fractions 2-3, indicating the dissociation of GnRH mRNA and the translation complex. A clear shift in the distribution of ribosomes associated with GnRH mRNA occurred after buserelin treatment, indicating that buserelin also decreases the translational efficiency of GnRH mRNA.

Discussion

The concept of ultrashort feedback mechanism was originally postulated by Martini and his coworkers who proposed that the synthesis, storage, and/or release of hypothalamic releasing hormones might be influenced by hypothalamic hormones present in the circulation (Hyyppa et al., 1971). Since then, several workers reported the autocrine regulation of GnRH on its own secretion (Bedran de Castro et al., 1985; Bourguignon et al., 1987; DePaolo et al., 1987; Naylor et al., 1989; Sarkar, 1987; Valenca et al., 1987; Zanisi et al., 1987). Despite the large body of evidence for autocrine inhibitory action of GnRH on its own secretion, little is known about the regulation of GnRH gene expression by GnRH itself. In this regard, we recently demonstrated that GnRH and buserelin, a strong GnRH agonist, decreases the GnRH promoter activity as well as GnRH mRNA level in a dose- and time-dependent manner, suggesting the presence of autoregulatory feedback mechanism operating at the level of GnRH gene transcription (Cho et al., 1997). In the present study, we further evaluated whether other control levels of GnRH gene expression can be affected by buserelin treatment. We found that buserelin decreases the stability as well as the translational efficiency of GnRH mRNA together with GnRH promoter activity. This indicates that the autoregulatory feedback mechanism operates at multiple control levels of GnRH gene expression in GT1-1 neuronal cells.

Results obtained by a pulse-chase experiment (Fig. 1) indicate that a relatively long lag time is required for buserelin-induced decrease in GnRH gene expression. While as short as 15 min exposure to buserelin is sufficient to decrease GnRH mRNA levels 24 h later (Fig. 1A), more than 12 h incubation is required to see the decrease in GnRH mRNA after buserelin treatment (Fig. 1B). This suggests that initial signals prompted by buserelin treatment induce a cascade of downstream reactions including the activation of other genes, which in turn decrease GnRH gene expression. One candidate signal to be prompted by buserelin treatment is the protein kinase C (PKC)-mediated pathway. Several

lines of evidence support this possibility. It was reported that GT1 cells express GnRH receptors (Krsmanovic et al., 1993), which eventually activate PKC when bound by its cognate ligands (Kaiser et al., 1997). Activation of PKC is well known to repress GnRH gene transcription (Bruder and Wierman, 1994; Gore et al., 1995; Sun et al., 1997). Cesnjaj et al. (1993) also demonstrated that GnRH or its agonist induce c-*fos* in GT1-7 cells, which has been linked to PKC-mediated repression of GnRH promoter activity (Bruder et al., 1996). Thus, it is quite plausible that the action of buserelin is mediated by PKC activation.

Recently, Roberts and his coworkers reported that GnRH mRNA turnover is also induced by phorbol 12-myristate 13-acetate (PMA), a potent PKC activator (Gore et al., 1997). They found that PMA causes GnRH mRNA half-life to decrease from 30 to 11 h, and that PMA treatment (4 and 8 h) results in a significant reduction in the length of the GnRH mRNA poly-(A) tail. Indeed, there is ample evidence that PMA induce destabilization of mRNA as a post-transcriptional regulation mechanism (Lee et al., 1994; Takahama et al., 1992). The present study demonstrated that buserelin also decreases the stability of GnRH mRNA. Under the basal condition, GnRH mRNA has a long half-life (22-30 h) as reported by others (Bruder and Wierman, 1994; Gore et al., 1997). In the presence of buserelin, however, the half-life of GnRH mRNA profoundly decreased to approximately 5 h (Fig. 2). The strong decrease in the stability of GnRH mRNA together with the decrease in GnRH promoter activity might explain the strong reduction in GnRH mRNA levels by buserelin treatment. Although the mediation of buserelin signal by PKC is highly plausible in many respects, it is still uncertain whether buserelin actually activates PKC in GT1-1 cells. Moreover, our preliminary study showed that calphostin C, a highly specific PKC inhibitor, cannot block buserelin-induced decrease in GnRH gene expression (data not shown). Thus, it is still an open question to examine which specific signaling pathway is involved in buserelin-induced decrease in GnRH gene expression.

The notion of indirect activation of other genes by buserelin treatment can be supported by the finding that actinomycin D can block buserelin-induced decrease in GnRH mRNA (Fig. 2). Since actinomycin D is a general transcription inhibitor (Sobell, 1985), it is likely that buserelin-induced decrease in GnRH mRNA level may be mediated by transcription of a gene(s) whose product(s) may be involved in the regulation of GnRH mRNA stability. In fact, the regulation of mRNA stability are believed to be dictated by specific determinants/ elements which may reside in the 5′-UTR, 3′-UTR, or the open reading frame domains of mRNA (Atwater, 1990). These determinants can interact with RNA binding proteins (known as *trans*-acting factors) to destabilize or stabilize the mRNA molecule, presumably by modifying accessibility of the transcript to nuclease attack (Atwater et al., 1990). Interestingly, our preliminary study showed that puromycin, a translation blocker, can partially block the inhibitory effect of buserelin on the GnRH mRNA level in GT1-1 cells (data not shown). Further studies are required to delineate the precise molecular mechanism by which buserelin reduces the stability of GnRH mRNA in these neuronal cells.

We previously reported strong decreases in the basal as well as K^+-induced GnRH secretion in GT1-1 cells treated with buserelin for 24 h (Cho et al., 1997). Moreover, cellular GnRH peptide contents are significantly reduced in buserelin-treated cells. In agreement with this finding, we observed in the present study that the translational efficiency of GnRH mRNA was also significantly reduced in buserelin-treated GT1-1 cells. While most GnRH mRNA from vehicle-treated GT1-1 cells was found in heavy fractions, the peak GnRH mRNA from 12 h of buserelin-treated GT1-1 cells was observed in the medium-sized fractions (Fig. 3). A clear shift in the distribution of ribosome-associated GnRH mRNA indicates that buserelin also decreases the translational efficiency of GnRH mRNA. The translational control of GnRH gene expression is yet poorly understood. Only recently, one report by Gore et al. (1995) showed that phorbol esters negatively regulate the translational efficiency of GnRH mRNA. The molecular mechanism underlying translational control of GnRH gene expression remains unknown and needs to be explored.

In conclusion, the present study clearly demonstrates that GnRH can exert autocrine regulation at multiple control levels of gene expression, such as mRNA stability and translational efficiency together with gene transcription.

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